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The Integument of the Marine Echiuran Worm *Urechis caupo*

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Abstract. During low tide, the burrow water of the marine echiuran worm *Urechis caupo* becomes hypoxic, and hydrogen sulfide concentrations reach levels that would be toxic to most animals. Integument morphology in *U. caupo* is evaluated as an exchange surface and as a permeation barrier. Adaptive features include the rugose nature of the epidermis, which increases the surface area for oxygen uptake, and the thick muscular body wall, which provides a chief motive power in creating peristaltic movements along the body wall to ventilate the burrow. The epidermis is covered by a cuticle and contains two types of mucus-secreting cells: orthochromatic and metachromatic. Underlying connective tissue and three muscle layers form the bulk of the body wall. The integument does not present a significant structural barrier to permeation, although the mucus secreted by the epidermal cells may retard sulfide entry. Ultrastructural studies suggest three possible mechanisms that *U. caupo* may use to counteract the toxic effects of sulfide at the integumentary surface: metabolism of symbiotic bacteria embedded in the innermost cuticle layer and grouped together in the superficial epidermis, dying off of peripheral, sulfide-exposed cells, and oxidation of sulfide at specialized, iron-rich, lysosomal organelles termed sulfide oxidizing bodies.

Introduction

Invertebrate integuments are morphologically and biochemically diverse but, because they are located at the interface of the organism with its environment, have common adaptive and protective functions. They provide physical protection for internal organs, regulate the exchange of materials between the organism and its environment, and act as a barrier to the ingress of various environmental agents.

Environmental hydrogen sulfide, produced by both natural and industrial sources, poisons aerobic respiration by binding to cytochrome c oxidase (National Research Council, 1979). No marine animal has been shown to be impermeable to ambient sulfide, and a variety of sulfide detoxification mechanisms have been reported (reviewed in Somero *et al.*, 1989), including the secretion of a mucus coat that buffers sulfide entry into the priapulid worm *Halicryptus spinulosus* (Oeschger and Janssen, 1991).

Although sulfide levels less than 1 μM are fatal to most organisms, the marine worm *Urechis caupo* lives in mud and sand flats with sulfide levels of 25–30 μM (Arp *et al.*, 1992) and is freely permeable to sulfide (Julian and Arp, 1992). The cytochrome c oxidase activities of the tissues of *U. caupo* are as sensitive to sulfide as homologous enzymes of other animals (Powell and Arp, 1989), indicating that this worm must either protect sensitive cytochromes in the body wall integument or tolerate anaerobic metabolism. Eaton and Arp (1993), however, have demonstrated that living worms rely primarily on aerobic respiration when exposed to sulfide, and various physiological adaptations that enable sulfide tolerance and detoxification have been reported (Arp *et al.*, 1992; Menon and Arp, 1992a,b; Arp, 1991; Powell and Arp, 1989).

Several early studies of the body wall of *U. caupo* (Newby, 1941) and other echiuran worms (Jameson, 1899; Jose, 1964) provide only limited information about histology and none, of course, about the ultrastructure of the cells that constitute the integument. The intent of this study was to examine the structure and histochemistry of the integument of *U. caupo* and, in particular, to evaluate its role as a protective organ or exchange surface in an animal living in the physiologically challenging environment of the marine mudflat. We report ultrastructural

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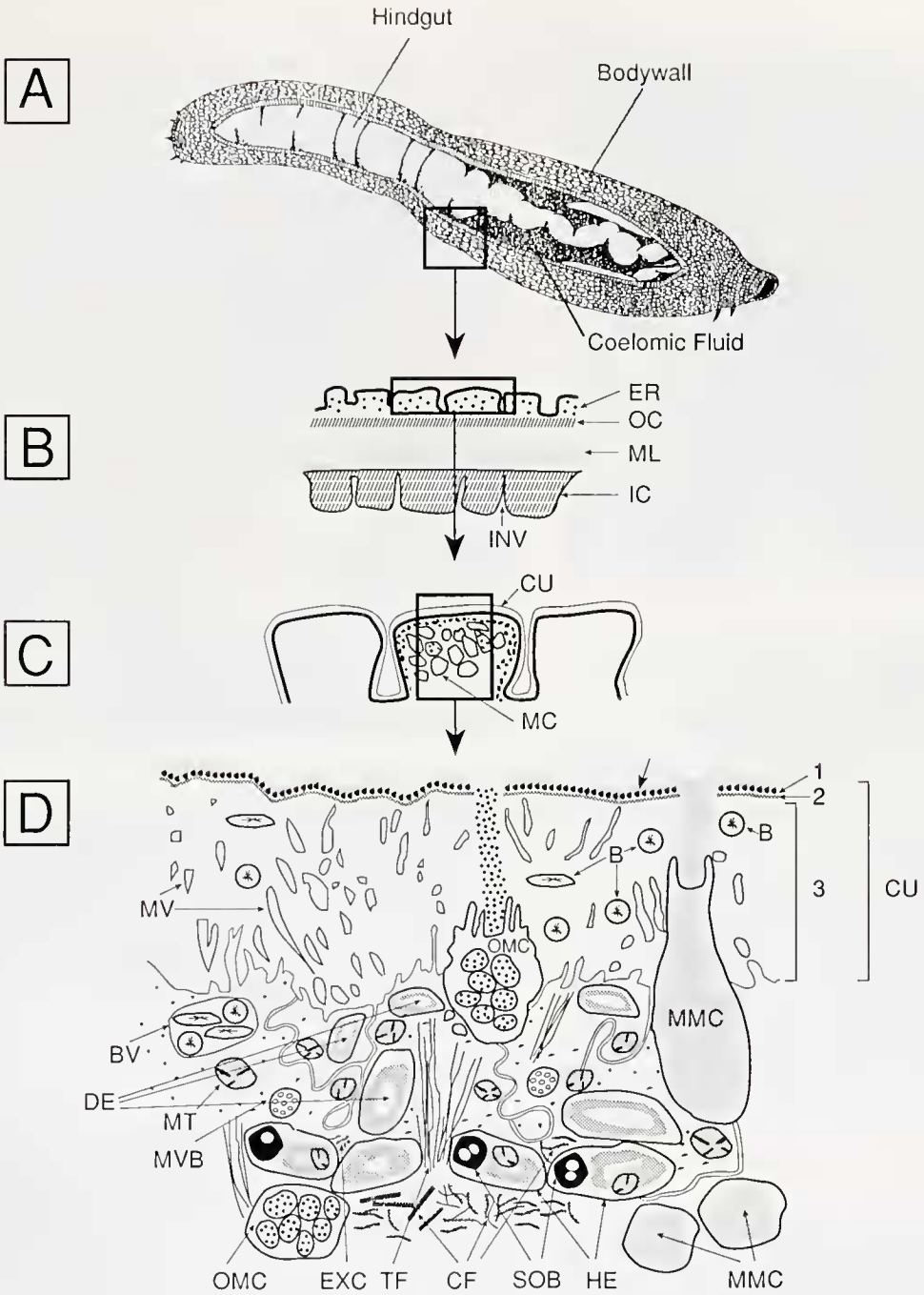
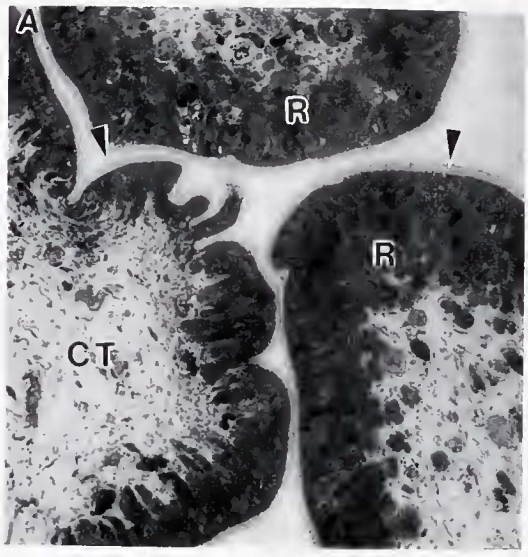


Figure 1. Schematic diagram of the gross anatomy and body wall structure of *Urechis caupo*. (**A**) Body plan and internal anatomy. (**B**) Schematic diagram of a 5 μm thick paraffin section of the body wall showing rugose epidermis (ER) followed by three muscle layers: outer circular muscle layer (OC), middle longitudinal muscle layer (ML), and innermost circular muscle layer (IC) showing invaginations (INV) at regular intervals. (C) Schematic representation of 0.5–1.0 μm thick plastic section showing rugae of the epidermis with mucus cells (MC) covered by a thin cuticle (CU). (**D**) Schematic representation of the ultrastructure of the epidermis at a magnification of about 13,000. Note that the cuticle (CU) is made up of three layers: (1) an outer layer consisting of epicuticular projections covered by fuzzy mucus (arrow) derived from metachromatic mucus cells (MMC), (2) a middle layer that is thin and granular, and (3) an innermost layer, which is the largest, and contains many microvilli (MV) and bacteria (B). Two types of mucus cells are evident in the epidermis: orthochromatic (OMC) and metachromatic (MMC). Groups of bacteria occur inside vacuoles (BV) in the superficial epidermal layers. Numerous mitochondria (MT), tonofilaments (TF), and multivesicular bodies (MVB) are present in the deeper layers of the epidermis. Healthy epidermal cells (HE) often have lysosomal sulfide oxidizing bodies (SOB) which possess empty vacuoles. Dying epidermal cells (DE) show condensed cytoplasm and a loss of organelles. The extracellular matrix (EXC) is composed of collagen fibers (CF) with a beaded appearance.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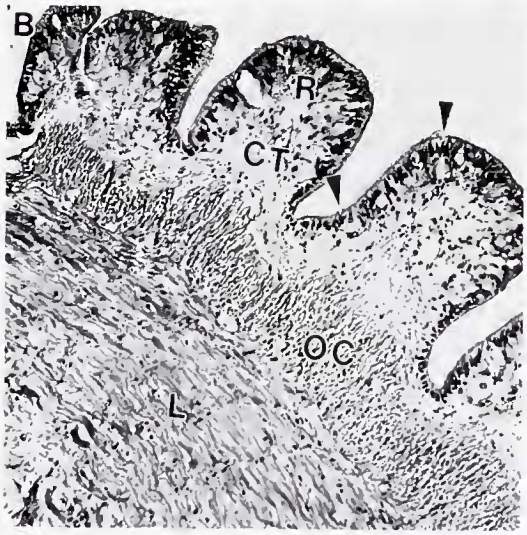
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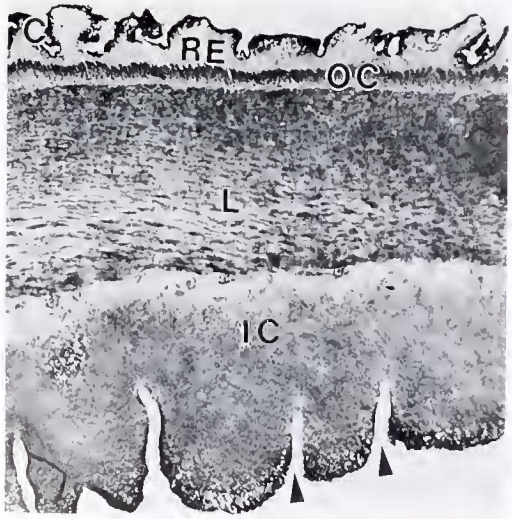
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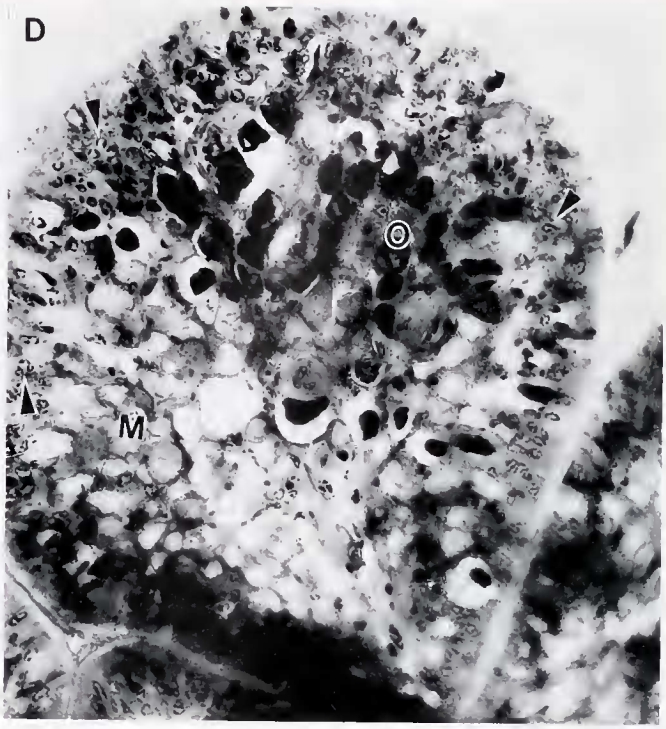
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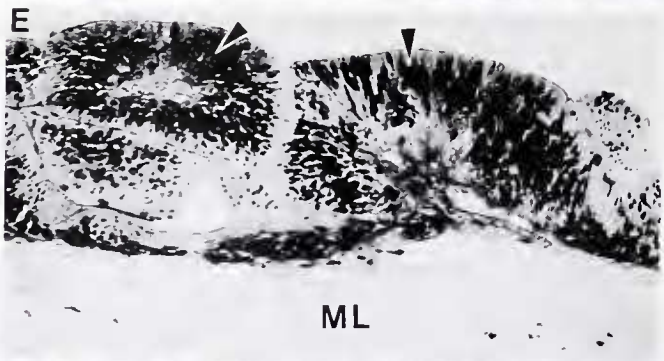
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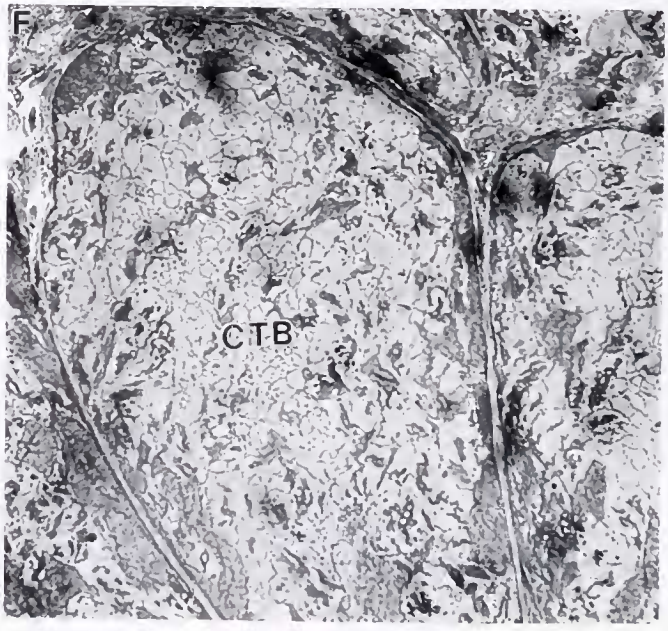
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evidence suggesting that *U. caupo* may protect the integumentary surface from the toxic effects of sulfide by secretion of mucus, metabolic activity of symbiotic bacteria, dying-off of peripheral, sulfide-exposed cells, and oxidation of sulfide at specialized lysosomal organelles.

Material and Methods

U. caupo individuals weighing 25 to 55 g were collected from Princeton Harbor, Elkhorn Slough, and Bodega Bay, California, during low tides. Animals were treated in one of three ways and then examined. Freshly collected worms were used within one day of capture from the natural environment. Laboratory-maintained worms were kept for 2 weeks to 4 months on the bottom of aerated aquaria free of sediment. These worms were not housed in artificial tubes, which has no effect on survival rate in the laboratory (authors' unpub. obs.). Sulfide-exposed worms were placed in respirometers, and oxygenated seawater was circulated through the chamber. Sulfide from a deoxygenated 8 m*M* sodium sulfide ($Na_2S \cdot 9H_2O$) stock solution, buffered with 125 m*M* imidazole and titrated with concentrated HCl to a final pH of approximately 6.8, was introduced with a peristaltic pump into the line just before the respirometer. Sulfide and oxygen levels in the respirometer were continuously monitored with electrodes that led to a data collection system (Arp *et al.*, 1992; Eaton and Arp, 1993).

Prior to dissection, the coelom of each animal was injected with muscle relaxant (0.4 *M* $MgCl_2$, 0.5 ml/g body weight), and the animal was left in water containing 0.4 *M* $MgCl_2$ until it had completely relaxed. Once relaxed, the body wall was cut open through a longitudinal incision in the mid-dorsal line. For light microscopy, body wall samples from three different regions were taken: the anterior region just behind the mouth, the middle region, and the posterior region. The tissues were fixed in either Bouin's fixative or 10% formalin for 24 h, dehydrated in an ethanol series (70%, 100%), cleared in toluene, and embedded in paraffin. Thick sections (5 μm) were cut, mounted on slides, and stained with hematoxylin and eosin for routine histology.

For electron microscopy, body wall samples were taken from the mid-dorsal region and fixed in 3% glutaraldehyde in 0.1 *M* cacodylate buffer, with 0.4 *M* NaCl to maintain the proper osmolarity (Holland and Nealson, 1978). Three 10-min rinses were carried out in the same solution without glutaraldehyde. Postfixation was for 30 min in 1% osmium tetroxide in 0.1 *M* cacodylate buffer (pH 7.3) with 0.4 *M* NaCl. All samples were rapidly dehydrated in an ethanol series (50%, 70%, 95%, 100%), and specimens were transferred to propylene oxide and embedded in Eponate 12 resin (Ted Pella). Semi-thin sections (0.5–1.0 μm) were stained with toluidine blue for light microscopy. Sections in the pale gold to silver range were cut with a diamond knife on a Sorvall MT2 ultramicrotome, double stained with uranyl acetate and lead citrate, and observed under a Zeiss EM 10 operating at 60 kV.

Bacterial densities in the cuticles of two worms from each of the following treatments were estimated: freshly collected worms from Bodega Bay; laboratory-maintained animals in sulfide-free water; and sulfide-exposed worms in the laboratory. One grid was taken from each specimen, and 15 electron micrographs of different areas were photographed at the same magnification. Bacteria observed in photomicrographs at a final magnification of 32,000 were counted.

To demonstrate mucopolysaccharides, the following histochemical methods were performed with appropriate controls (Bancroft and Stevens, 1977). Paraffin sections were stained with periodic acid-Schiff's (PAS) for mucopolysaccharides; diastase treatment followed by PAS for glycogen; PAS in conjunction with alcian blue 8GX to distinguish neutral mucins from acidic mucins; alcian blue staining at pH 0.5 and 2.5 to confirm the sulfated and carboxylated mucins; methylation and saponification with alcian blue (pH 2.5) and critical electrolyte concentration with alcian blue (pH 2.5) to differentiate sulfated from carboxylated mucopolysaccharides; and toluidine blue and azure A for showing metachromasia and acidic mucopolysaccharides.

After calibration with a stage micrometer, an ocular micrometer and a whipple disc were used on the micro-

Figure 2. (**A**) Tangential section of epidermal rugae (R). Epidermal cells covered by a cuticle (arrows) and stained with PAS showing the presence of mucopolysaccharides. Underlying connective tissue (CT) does not show PAS positive reaction. 1 μm Epoxy section. ×220. (**B**) Transverse section of epidermis showing rugae (R), underlying connective tissue (CT), and the outer circular (OC) and middle longitudinal (L) muscle layers. Note darkly stained nuclei on the periphery of epidermis (arrows). 5 μm paraffin section. Hematoxylin and eosin stain. ×110. (**C**) Transverse section of the body wall showing rugose epidermis (RE), followed by three muscle layers: outer circular (OC), middle longitudinal (L) and inner circular (IC). The innermost layer shows deep invaginations (arrows) at regular interval. 10 μm frozen section. Hematoxylin and eosin. ×20. (**D**) Epidermal rugae at a higher magnification. Two types of mucus-secreting cells are evident: orthochromatic (O), which are darkly stained, and metachromatic (M) showing a fine reticulate pattern. Note cells with pyknotic nuclei at the periphery of the epidermis (arrows). 1 μm Epoxy section. Toluidine blue stain. ×220. (**E**) Transverse section of epidermis showing the presence of acidic mucopolysaccharides in the rugae (arrows). Underlying muscle layers (ML) do not react with stain. 5 μm paraffin section. Alcian blue stain. ×110. (**F**) Transverse section passing through the anterior region of the body at the proboscis showing connective tissue bundles (CTB). 1 μm Epoxy section. Toluidine blue. ×570.

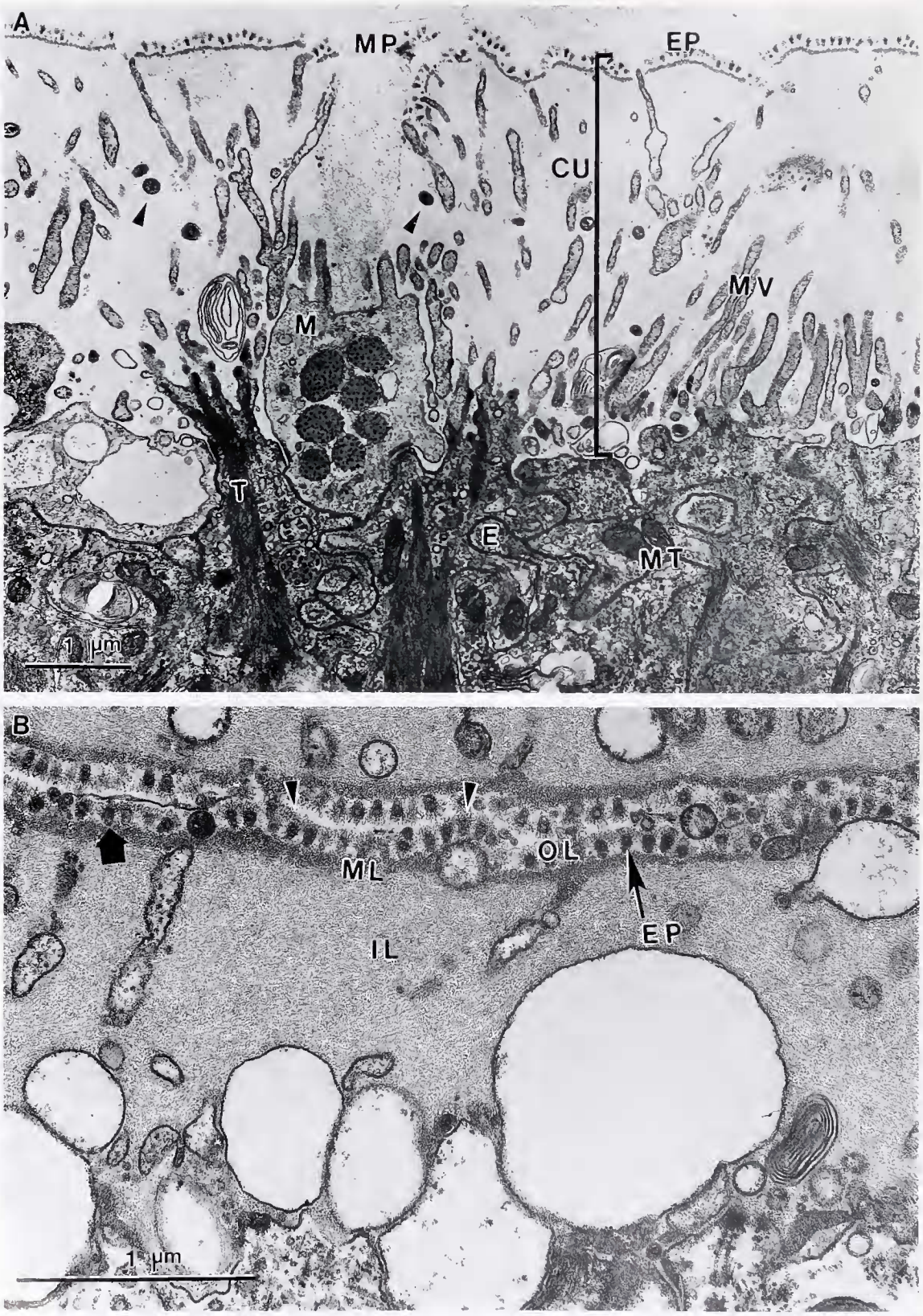


Figure 3. (A) Overview of the epidermis showing the cuticle (CU) with epicuticular projections (EP) in the outermost layer, scattered bacteria (arrows) and microvilli (MV) in the innermost layer, a mucus-secreting cell (M) discharging mucus through the mucus pore (MP), tonofilaments (T), and numerous mitochondria

scope to count the number of mucus cells per 0.2 mm^2 area in 0.5 to 1.0 μm thick plastic sections of the epidermis of freshly collected and laboratory-maintained animals (Gona, 1979). The terminology of Gona (1979) for the color of mucus cells after staining with toluidine blue has been adopted. Counts were made from three to five mid-dorsal body regions of animals. Dying epidermal cells in sulfide-free (laboratory-maintained) and sulfide-exposed (freshly collected and exposed to sulfide in the laboratory) animals were distinguished under the light microscope by their intensely basophilic nuclei and loss of cytoplasm, and were counted. The body wall epidermis of sulfide-exposed animals was grazed with a sharp razor blade in the mid-dorsal region prior to sulfide exposure, and the mucus or dying cells in the sample counted (control). The worms were then allowed to heal in the aquaria for 4 to 5 days, and were then placed in a flow-through aquarium with oxygenated seawater containing 50 to 70 μM sulfide for 48 to 78 h (Arp *et al.*, 1992). A second epidermal sample was taken about 1 cm from the original sample and the cells counted (experimental).

Results are presented as the mean ± the standard deviation of the mean. Statistical tests were accepted as significant for $P < 0.05$. Fixed effects analysis of variance (ANOVA) was employed to test for significant differences between animal treatments (Zar, 1984).

Results

An overview of the structure of the integument of *U. caupo* is shown diagrammatically in Figure 1, at the light microscopic level in Figure 2, and at the ultrastructural level in Figure 3A. The surface of the body wall integument is traversed by fine irregular channels that form a rugose surface (Fig. 1B, 2B, C). The epidermis is covered by an outermost cuticle that harbors prokaryotic cells (Fig. 1D, 3A, 4). The epidermis contains two types of columnar mucus-secreting cells (Fig. 1D, 2D). Another type of cell present in the superficial layers of the epidermis is characterized by large, pyknotic, and intensely basophilic nuclei with chromatin material dispersed toward the nuclear membrane (Fig. 2D, arrows). Unusual cytolysosomes are present in many of the epidermal cells as well (Fig. 1D, 7, 8). Subjacent to the epidermis is a thin layer of connective tissue (Fig. 2A, B), and below it are three distinct muscle layers: an outer circular, a middle longitudinal, and an innermost circular muscle layer (Fig. 2C). The innermost layer shows invaginations at regular intervals (Fig. 2B, C, arrows). The anterior body wall in the region of the proboscis has additional connective tissue bundles (Fig. 2F), and the posterior body wall in the region of the anus has a thicker muscle layer than does the anterior and middle body wall.

Ultrastructural observations indicate that the integumentary cuticle consists of three layers. The outermost layer is composed of membrane-bound and regularly arranged epicuticular projections that show a definite substructure of an electron-dense lining surrounding a central core (Fig. 3A, B). The epicuticular projections arise from the exposed tips of the microvilli, which are cytoplasmic extensions of epidermal cell membranes and are present in the innermost layer of cuticle (Fig. 3A). Covering the epicuticular projections are fine strands of mucus material forming the supracuticular mucus coat (Fig. 3B). The fuzzy nature of this mucus material is ultrastructurally similar to the contents of the metachromatic mucus cells (see below), suggesting that this cell type is the origin of the mucus coat. The middle layer, on which epicuticular projections rest, is slightly electron dense (Fig. 3A, B). The innermost layer is the largest, and is composed of thin and loosely arranged fibers interspersed with abundant microvilli (Fig. 3A, B). Closely applied to the inner surface of the base of the microvilli is a prominent bundle of supporting tonofilaments that extend a considerable distance (Fig. 3A). Numerous mitochondria are concentrated in the most superficial epidermal layers (Fig. 3A).

Extracellular, rod-shaped cells of a structure typical of gram-negative bacteria occur scattered between the tips of microvilli in the cuticular matrix (Fig. 3A, 4A, B), as well as in groups of 4 to 5 enclosed in vacuoles near the surface of the epidermis (Fig. 4A, C). Cuticular and epidermal bacterial are similar in size, ranging from 0.22 to 1.80 μm. In both cases, the bacterial nature of these cells is confirmed by the presence of a typical diffuse reticulum of DNA threads, the absence of a distinct nucleus, and by the double cell wall with an inner amorphous zone (Fisher, 1990). Bacterial densities vary significantly depending upon treatment ($P < 0.05$). Freshly collected worms from Bodega Bay, California (a high sulfide site; Arp *et al.*, 1992) have high bacterial densities (8.97 ± 2.48), laboratory-maintained worms have the lowest bacterial densities (5.67 ± 0.99), and worms exposed to 50 to 70 μM sulfide in the laboratory for 48 to 72 h have the highest bacterial densities (15.07 ± 1.98).

The mucus-secreting cells of the epidermis are large and ovoid. Based on their staining properties with the metachromatic stains toluidine blue and azure A, two

(MT) in the epidermal cells (E). (**B**) Higher magnification showing details of the cuticle lining of two adjacent rugae. The outermost layer (OL) shows epicuticular projections (EP) with an electron-dense lining surrounding a central core (arrow). This layer is covered with a fuzzy mucus coat (arrow heads), followed by a thin and electron-dense middle layer (ML), and largest innermost layer (IL) made up of thin fibers.

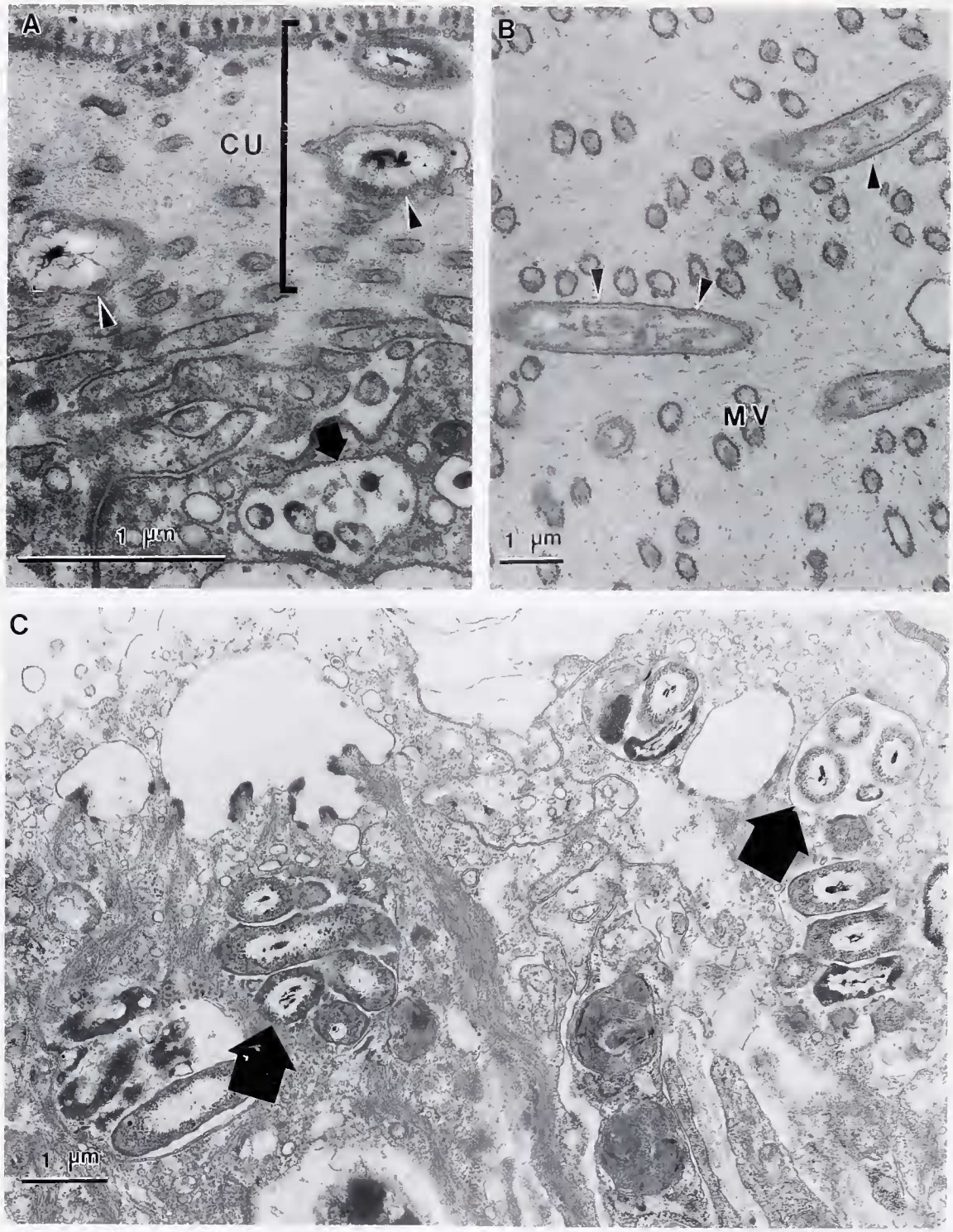


Figure 4. (**A**) Ultrastructure of the cuticle (CU) and part of the superficial epidermis with solitary bacteria (arrow heads) in the innermost layer of the cuticle and a group of bacteria within a vacuole (arrow) in the superficial epidermal layer. (**B**) Higher magnification reveals the double cell wall (arrow heads) of solitary bacteria and diffuse chromatin material with microvilli (MV) cut in cross sections. (**C**) Epidermis of a sulfide-exposed worm showing abundant bacteria grouped in vacuoles (arrows) in the outermost region of the epidermis.

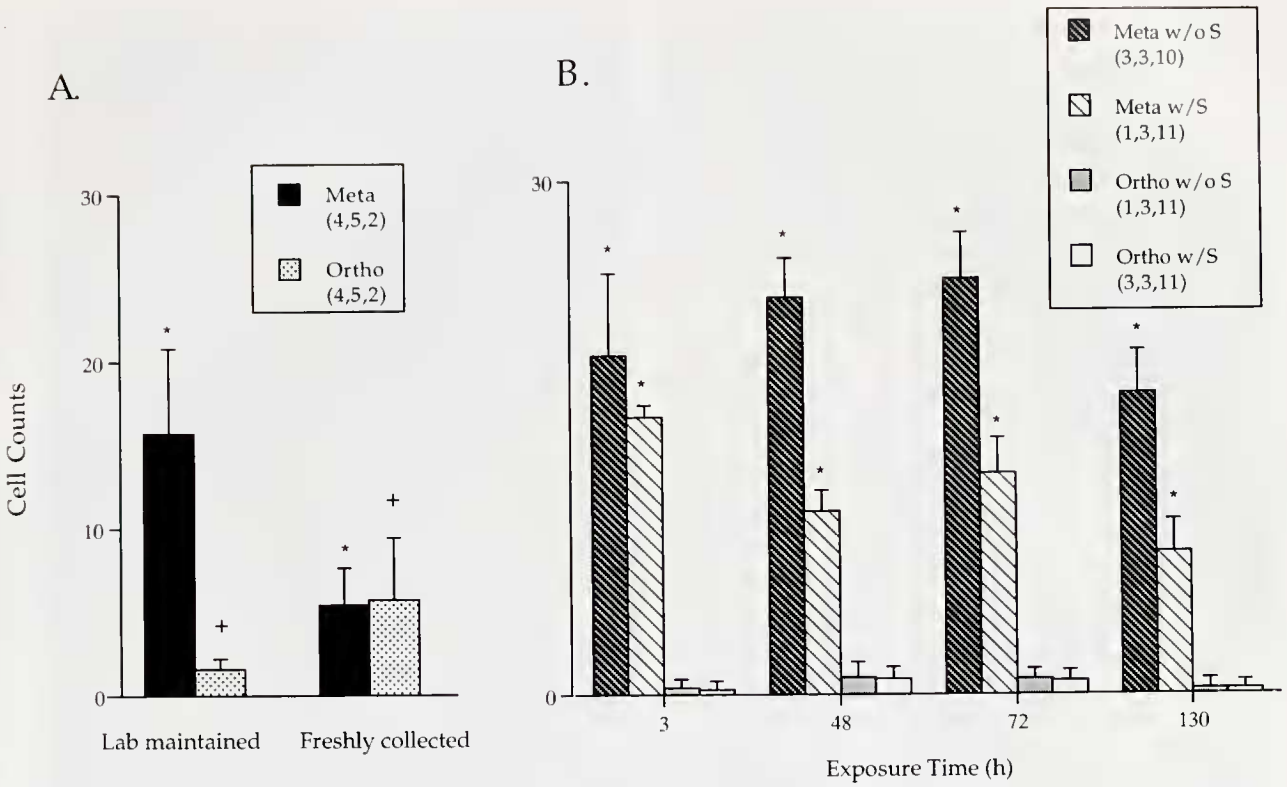
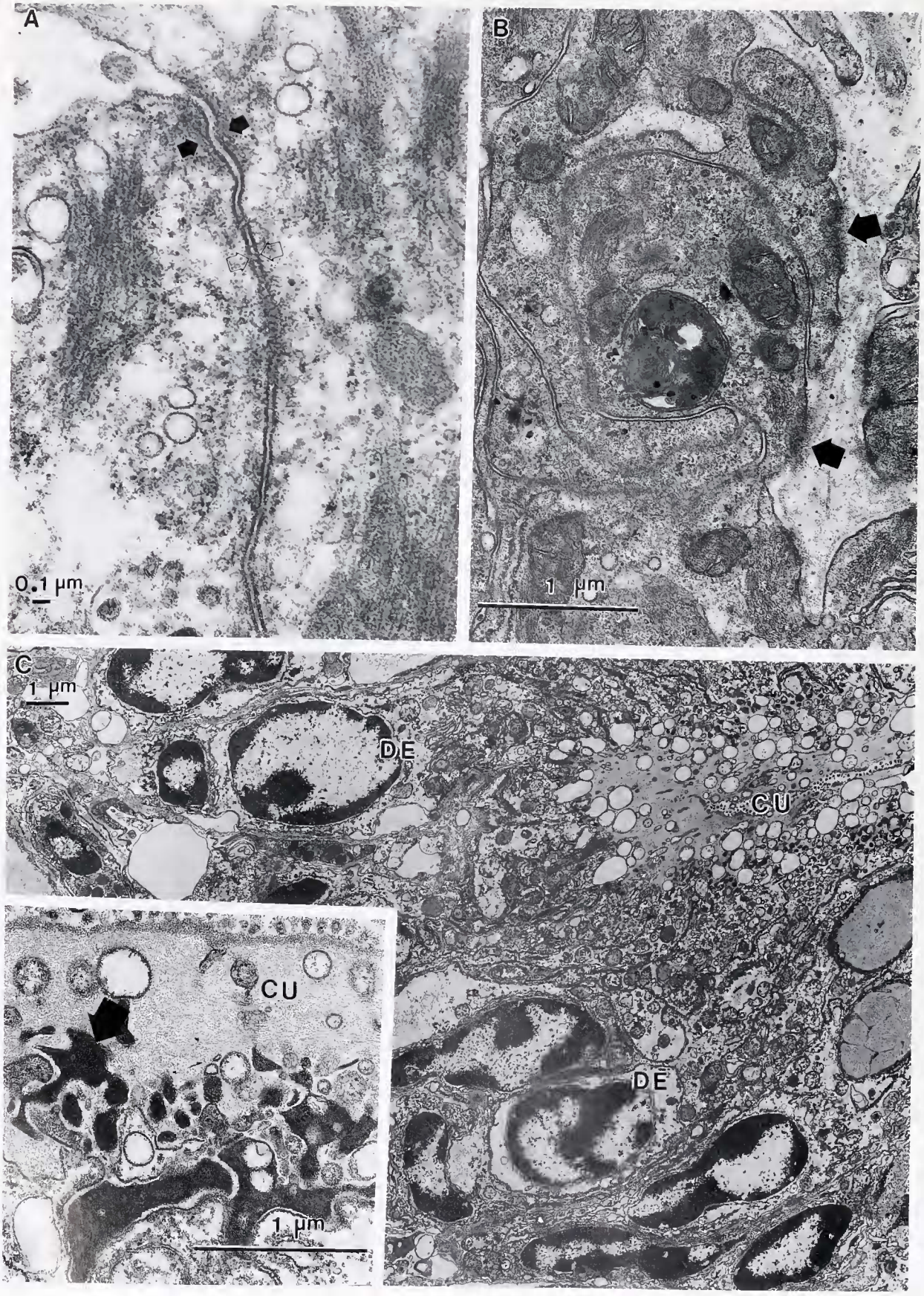


Figure 5. The number (mean ± SD) of mucus cells in the epidermal surface of *Urechis caupo* present in a 0.2 mm^2 field under light microscopy. (**A**) The number of mucus cells in the epidermal surface of freshly collected animals and animals maintained in sulfide-free seawater in the laboratory for two to three weeks. (**B**) The number of mucus cells in the epidermal surface of animals maintained in sulfide-free seawater in the laboratory for one to four months, compared to the number of mucus cells in the epidermal surface of the same animals after exposure to 45 to 110 μM sulfide in the laboratory. Metachromatic cell counts from freshly collected animals and sulfide-exposed animals were significantly lower than those of the laboratory-maintained animals, and orthochromatic cell counts from freshly collected animals were significantly higher than those of the laboratory-maintained animals. Orthochromatic cell counts from laboratory-maintained animals were not significantly altered with sulfide exposure. Numbers in parentheses are the number of worms sampled, followed by the number of body regions examined, and the number of fields counted per region. Data bars with like symbols (asterisks and crosses) are significantly different ($P < 0.05$).

types of mucus-secreting cells are evident: orthochromatic mucus cells identified by the presence of discrete blue granules, and metachromatic cells identified by the presence of pink strands or a very fine reticulum within the mucus cell cavity. Animals freshly collected from the environment during low tide had very few purple to pink metachromatic cells filled with mucus (5.4 ± 2.2; Fig. 5A) and more empty metachromatic cells (14.1 ± 5.4; data not shown). Animals maintained in sulfide-free seawater in the laboratory for 3–4 weeks had significantly higher numbers of full metachromatic cells than did freshly collected worms (15.7 ± 5.1; Fig. 5A, $P < 0.05$) and negligible numbers of empty metachromatic cells. Sulfide exposure in the laboratory resulted in a significant decrease in the number of metachromatic cells at all exposure times (Fig. 5B; $P < 0.05$; the initial numbers of metachromatic cells in this group are higher than the laboratory-maintained group in Figure 5A because these animals had been kept in the laboratory longer, 2–4 months). Orthochromatic mucus-secreting cell counts in animals maintained in sulfide-free seawater in the laboratory for 3–4 weeks were significantly lower (1.6 ± 0.6; Fig. 5A) than cell counts from freshly collected worms (5.7 ± 3.7; $P < 0.05$; Fig. 5A). However, animals exposed to sulfide in the laboratory showed no significant change in the number of orthochromatic cells (Fig. 5B). Animals collected from the other two sites revealed similar patterns in the distribution and type of mucus-secreting cells (data not shown).

Histochemical techniques employed to distinguish the types of mucopolysaccharides indicate the presence of neutral and acidic substances in the epidermis. When PAS is used in conjunction with alcian blue, the epidermal cells stain with alcian blue and do not respond to the subsequent PAS treatment, thus distinguishing the neutral mucin from acidic mucins. The acid moiety of mucus is composed of carboxyl or sulfated groups, or a combination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of both, and these groups were distinguished by the following tests. Strong reactions with alcian blue at pH 2.5 indicate the presence of both carboxylated and sulfated mucopolysaccharides. Furthermore, positive staining at pH 0.5 indicates the presence of sulfated mucins. The critical electrolyte concentration-alcian blue staining at low molarity (0.06 *M*) gave an intense reaction, indicating the presence of both types of acidic mucins (Scott and Dorling, 1965). At higher molarity (0.5 *M* and 0.9 *M*), the epidermal cells yield a positive reaction, again showing the presence of sulfated mucins. The restoration of alcian blue staining, which occurred after high temperature combined with methylation and then saponification, also indicates the presence of carboxymucins (Spicer and Lillie, 1959). With the metachromatic dyes, toluidine blue and azure A, certain cells of the epidermis appear to be strongly metachromatic, indicating the presence of acidic mucus.

The epidermis of *U. caupo* contains three junctional specializations: (1) zonula adherens are typically present at the cuticular surface of epidermal cells (Fig. 6A); (2) septate intercellular junctions, which have less distinct septa traversing the cleft between epithelial cells, lie basal to zonula adherens (Fig. 6A); and (3) hemidesmosomes, which consist of plaques of electron-dense cytoplasm, may be present anywhere from zonula adherens to extracellular matrix (Fig. 6B). A prominent extracellular matrix, underlying the epidermis, consists of fine fibrils of collagen but is not organized into layers. Fibrils are oriented in all directions and do not display prominent cross-banding, although they have a slightly beaded appearance (not shown here).

Another cell type is present in the superficial layers of epidermis immediately below the cuticle of *U. caupo* (Figs. 6C, 7A, 8). In freshly collected worms, these cells exhibit the following characteristics of cell death: *e.g.*, a large nucleus-cytoplasmic ratio, condensed and vacuolated cytoplasm, chromatin material aggregated in large compact masses that border the nuclear membrane, and the loss of cellular organelles (Figs. 6C, 8). In some areas, chromatin or cytoplasmic material is broken down into fragments and appears to be in the process of extrusion from the cell (Fig. 6C, inset). Similar cells from the same tissue from laboratory-maintained animals, or deeper within the tissues of freshly collected worms, appear healthy, with visible cell organelles and less electron-dense chromatin material (Figs. 7A, 8). Dying cells, distinguished at the light microscopic level by their intensely basophilic nuclei and loss of cytoplasm, were counted in a similar manner to mucus cells. Freshly collected animals from all three sites had significantly higher numbers of dying cells than laboratory-maintained animals (33.52 ± 3.01 and 17.02 ± 2.99 per 0.2 mm^2 area, respectively; n = 10 worms, 5 regions per worm, 1 area per region; $P < 0.05$), and laboratory-maintained animals exposed to 50 to 70 μM sulfide for 48 to 78 h had significantly higher numbers of dying cells than before the exposure (32.65 ± 2.98 and 15.20 ± 1.36 per 0.2 mm^2 area, respectively; n = 4 worms, 5 regions per worm, 1 area per region; $P < 0.05$).

The cytoplasm of healthy superficial epidermal cells contains abundant cytolysosomes that show a partially lamellated pattern (Figs. 7A, B; 8). These bodies are composed primarily of iron and silicate, possibly in a paracrystalline array that may be responsible for their lamellated appearance (authors' unpublished X-ray micro analysis). They often possess empty vacuoles characteristic of mineral deposits and are close to mitochondria and occasionally bacteria (Fig. 8 and inset). Additionally, groups of electron-dense, swollen mitochondria are often observed surrounding secondary lysosomes (Fig. 7B inset), and cytolysosome density increases in sulfide-exposed worms (authors' unpub. obs.).

Discussion

Adaptive features of the integument of *U. caupo* for life in a periodically hypoxic and sulfidic habitat include the rugose nature of the epidermis, which increases the surface area for oxygen uptake, and the thick muscular body wall, which provides a chief motive power in creating peristaltic movements along the body wall to drive ventilatory and feeding currents through the burrow. The integument does not present a significant structural barrier to permeation, although the mucus secreted by the epidermal cells may retard sulfide entry. Ultrastructural studies suggest three possible mechanisms that *U. caupo* may use to various degrees to counteract the toxic effects of this naturally occurring environmental poison at the integumentary surface: metabolism of symbiotic bacteria, dying off of peripheral, sulfide-exposed cells, and oxidation of sulfide at lysosomal sulfide oxidizing bodies.

Sulfide exists in marine systems predominantly as H_2S and HS^-. Both are highly permeable, small molecules that

Figure 6. (**A**) Adjacent epidermal cells display specialized junctions such as zonula adherens (closed arrows) and septate junctions (open arrows). (**B**) Hemidesmosomes showing electron-dense cytoplasmic plaques on either side of the cells (arrows). (**C**) Epidermal cells below the cuticle (CU) of a freshly collected animal (sulfide-exposed) with dying epidermal cells (DE) exhibiting condensed cytoplasm, no visible cellular organelles, and chromatin material aggregated in the nuclear periphery. Inset: ultrastructure of a dying epidermal cell below the cuticle (CU) showing disintegration of the chromatin or cytoplasmic material (arrow).

easily diffuse through membranes via paracellular or transcellular routes (Powell *et al.*, 1979; Julian and Arp, 1992). Permeation of an integument by some molecules can be prevented by morphologically identifiable barriers such as tight junctions, a fibrous or mineralized cuticle, and the presence of lipids (Lillywhite and Maderson, 1988). *U. caupo* has three types of intercellular junctions—zonula adherens, macula adherens (desmosomes), and septate junctions—structures similar to those reported in several invertebrates (Coggeshall, 1966; Tyler, 1984). Tight junctions have not been reported in invertebrate epidermis except for tunicates; septate junctions substitute for tight junctions which are comparatively leaky and offer low resistance to diffusion (Lillywhite and Maderson, 1988). These features may explain why the body wall of this worm presents no significant permeability barrier to sulfide. Additionally, the numerous mitochondria in the epidermal cells immediately below the cuticle suggests that there is active absorption and direct transport of substances from the environment (Richards and Arme, 1979, 1980; Jouin and Gaill, 1990).

The integument of *U. caupo* is composed of simple columnar glandular cells covered by a cuticle. Underlying the epidermis are connective and muscular tissues that form the bulk of the body wall. There are two types of glandular mucus-secreting cells histologically distinguished as orthochromatic (granular) and metachromatic (reticulate) that secrete mucus of an acidic and neutral nature. The cuticle, as revealed by its ultrastructure, is covered by a thin film of mucus derived from the metachromatic cells. The formation of sulfated mucopolysaccharides for the mucus coat in *U. caupo* may use sulfide present in the environment. Vetter (1985) reported the incorporation of sulfur into normal organic compounds, such as the sulfur amino acids, mucopolysaccharides and sulfolipids, in clams from sulfidic habitats.

Metachromatic cells in freshly collected (sulfide-exposed) worms are reduced in number relative to those in the sulfide-free animals, and distinct empty metachromatic cells are present; empty metachromatic cells were not detected in worms from any other treatment. These animals are likely to be exposed to sulfide in the environment and may have empty metachromatic cells due to the recent secretion of mucus in response to sulfide or other physiological or ecological factors such as burrow friction in the natural environment. Sulfide exposure in the laboratory resulted in a decrease in metachromatic mucus cells and a noticeable increase in mucus production (authors' unpub. obs.). These data suggest that mucus is secreted in response to sulfide exposure and may reduce sulfide entry. The priapulid worm *Halicryptus spinulosus* has been reported to detoxify sulfide by trapping it in an iron-containing mucus that is secreted by associated epibacteria and rapidly turns the body wall black (Oeschger and Janssen, 1991). Although mucus may interact with sulfide at the body surface in *U. caupo*, there is no color change or evidence of a sulfide-trapping mechanism. Orthochromatic mucus-secreting cells were significantly more abundant in freshly collected animals and less prevalent in sulfide-free or sulfide-exposed laboratory-maintained animals. These cells may be involved in mucus secretion for burrow lubrication and mating (Richards, 1974) and may not be used while the worm is maintained out of a burrow in the laboratory.

The innermost layer of the cuticle and superficial epidermal region harbors extracellular, rod-shaped cells of a structure typical of gram-negative bacteria. The bacteria often have electron-transparent regions similar to those reported by Cavanaugh *et al.* (1981) in *Riftia pachyptila* trophosome, and these regions may have contained sulfur inclusions prior to fixation. A preliminary assay for the primary enzyme responsible for carbon dioxide fixation, ribulose-1,5-biphosphate carboxylase-oxygenase (RuBPCase), showed mean activity levels of 0.0018 IU/g in homogenized samples of *U. caupo* body wall (pers. comm. A. E. Anderson, Oregon State University). These levels are similar to those reported in pogonophoran tube worms, the bivalve *Calyptogena elongata* and several thyasirid clams (Somero *et al.*, 1989). Although further biochemical data are needed, the close proximity of bacteria to the external environment and the preliminary evidence of RuBPCase activity suggest that the symbionts metabolize chemoautotrophically, fueled by low levels of reduced sulfur compounds from the environment.

The extracellular location and size of the bacterial symbionts in *U. caupo* are similar to those described from several marine invertebrates from mildly reducing sediments (Felbeck *et al.*, 1983; Fisher, 1990). Their consistent abundance under the cuticle of *U. caupo* indicates a significant functional relationship. Symbiont-containing hosts usually possess a reduced digestive system (Fisher, 1990), but *U. caupo* not only has bacterial symbionts, but also a well-differentiated alimentary canal and an elaborate filter-feeding behavior (Fisher and MacGinitie, 1928; Judd and Arp, 1992). Thus, these worms are not sulfide-dependent; their symbionts probably provide sulfide detoxification rather than significant nutrition (Powell and Somero, 1986; Gaill *et al.*, 1988).

Epidermal cells situated just below the cuticle in sulfide-exposed individuals of *U. caupo*, appear to be dying, whereas the epithelial cells of laboratory-maintained worms, or cells from the deeper layers of the epidermis, appear healthy. These dying cells may be in the process of controlled cell death, or apoptosis, as characterized by the loss of cell organelles, condensation of the cytoplasm and nuclear chromatin, followed by fragmentation of the nucleus (Ojcius *et al.*, 1991). Dying cells in *U. caupo* go through two distinct deletion stages characteristic of apoptosis: nuclear and cytoplasmic disintegration, followed by shedding from epidermal surfaces as described

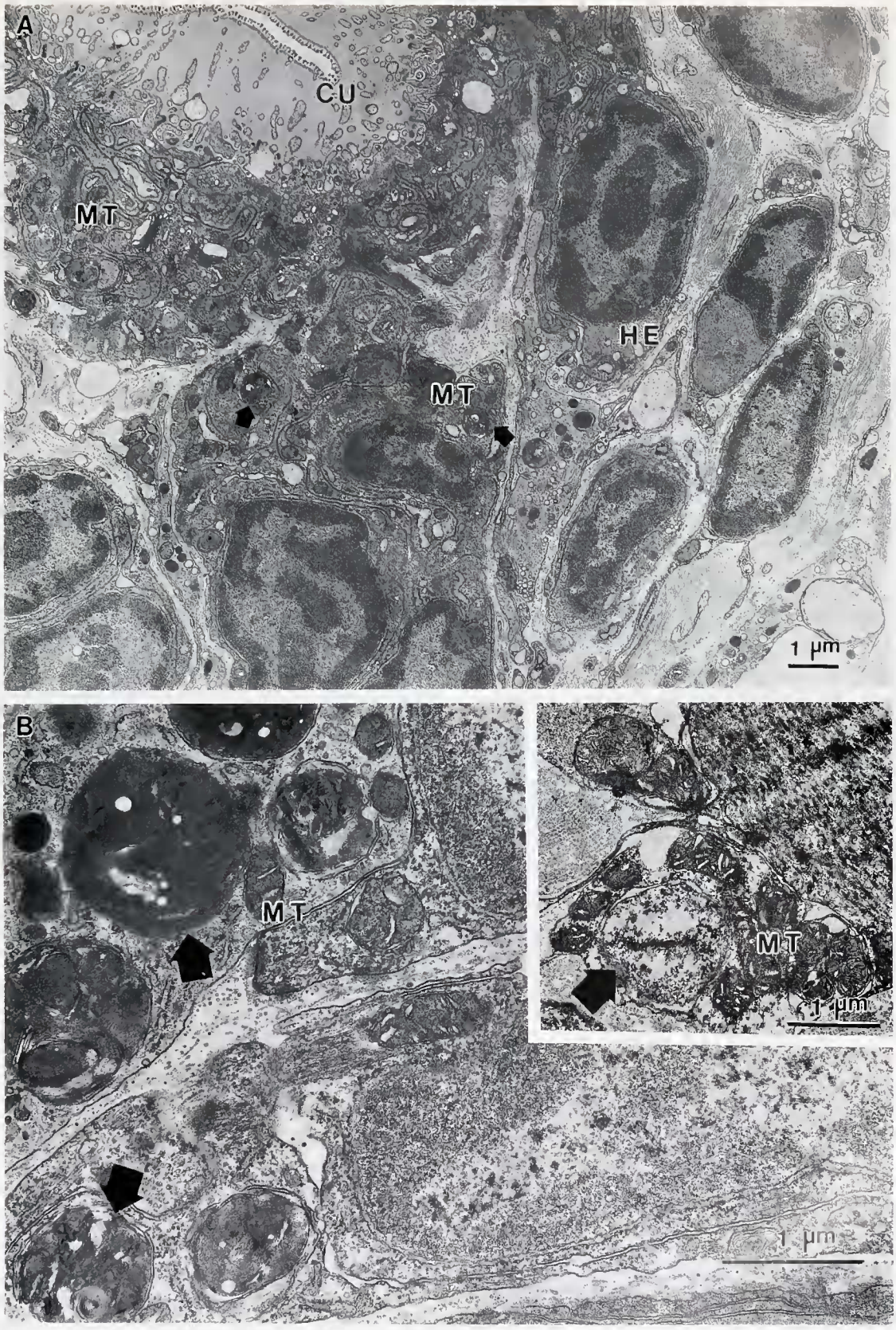

Figure 7. (**A**) Epidermis from a laboratory-maintained animal showing healthy epidermal cells (HE) below the cuticle (CU) with numerous mitochondria (MT), cytolysosomes (arrows), and a fair amount of cytoplasm. (**B**) Ultrastructure of epidermal cells showing numerous lysosomal sulfide-oxidizing bodies (arrows) and mitochondria (MT). Inset: ultrastructure of mitochondria from a sulfide-exposed worm showing altered morphology and close proximity to a cytolysosome (arrow).

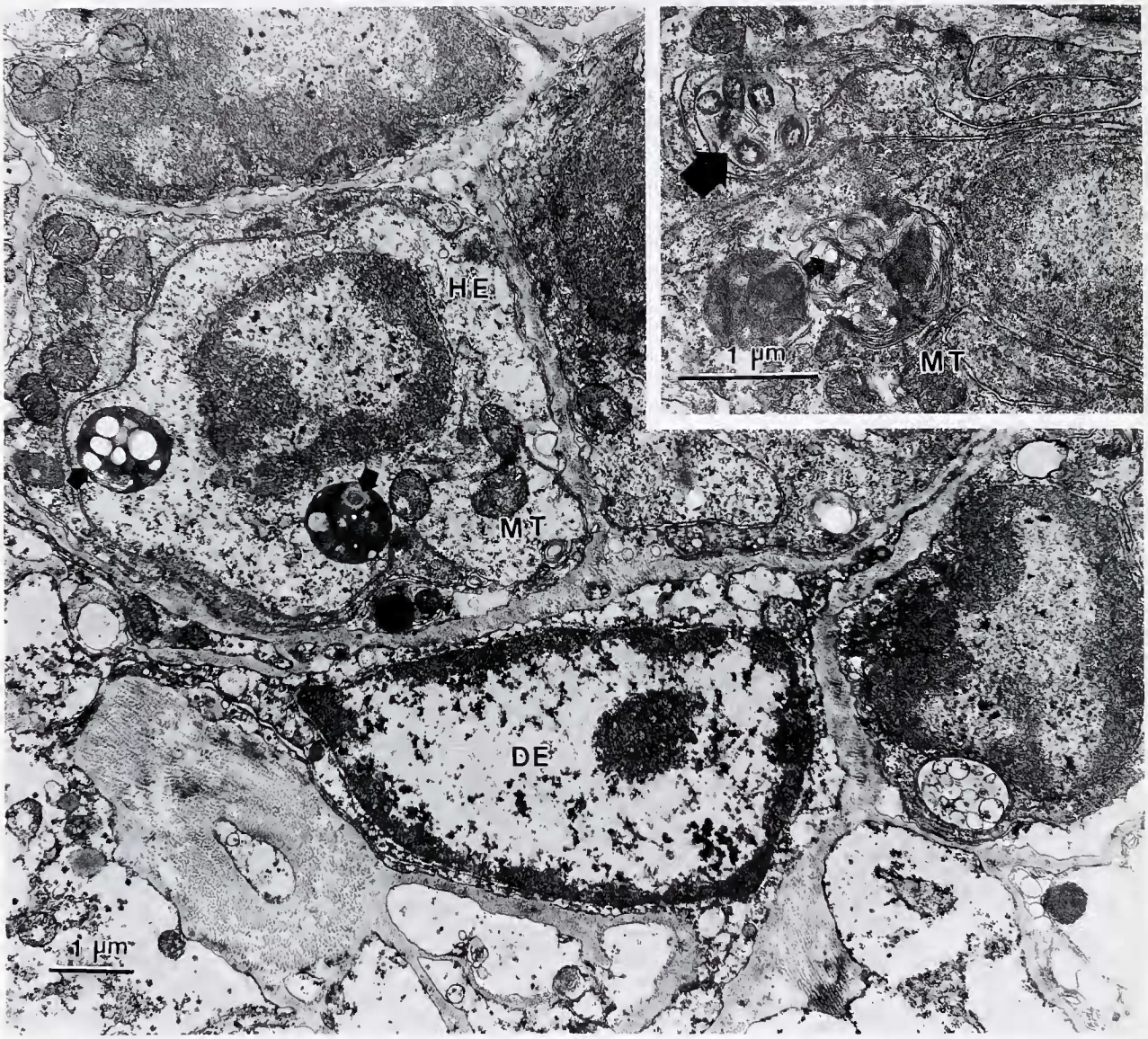

Figure 8. Epidermal cells from the deeper layers of body wall showing a few healthy cells (HE) containing lysosomal sulfide oxidizing bodies with inclusions (arrows) in close proximity to mitochondria (MT), and a dying cell (DE) with a pyknotic nucleus, condensed cytoplasm, and loss of organelles. Inset: higher magnification of the lysosomal sulfide oxidizing bodies showing inclusions (small arrow) in close proximity to mitochondria (MT) and bacteria (large arrow).

by Young (1987) in sunburned mammalian skin. Changes in intracellular concentrations of smaller molecules or ions (possibly sulfide molecules in *U. caupo*) have been shown to trigger apoptosis (Wyllie *et al.*, 1980). Differences in the number of dying cells between freshly collected animals, animals exposed to sulfide in the laboratory, and animals maintained in a sulfide-free environment in the laboratory, suggest that these cells increase in number in response to sulfide. This suggests that sulfide-sequestering subcuticular epidermal cells of *U. caupo* may die and be sloughed-off, thus eliminating sulfide from the organism. Alternatively, rapid cell turnover may simply be a nonspecific response to sulfide toxicity (rather than a discrete sulfide elimination mechanism) that occurs on the outside, or sulfide-exposed, surface of the animal's body, or cells may be dying in response to the mechanical abrasion associated with the burrow habitat of the animal.

Healthy epidermal cells lying below the dying cells contain structures morphologically similar to specialized organelles termed sulfide-oxidizing bodies. Sulfide oxidizing bodies were originally observed and described in *Solemya reidi* by Powell and Somero (1985) and have

also been reported in the hindgut of *U. caupo* (Menon and Arp, 1992b). These specialized lysosomal structures are predominantly composed of iron and often lie in close proximity to mitochondria and bacteria, suggesting a possible connection to sulfide-driven mitochondrial ATP production or bacterial nutrition (Felbeck, 1983; Powell and Somero, 1985, 1986; O'Brien and Vetter, 1990; Oeschger and Vetter, 1992).

Mitochondria in the epidermis of *U. caupo* collected from high sulfide environments or exposed to sulfide in the laboratory are unusually electron dense, swollen, and clumped together near cytolysosomes, and cytolysosome density increases in sulfide-exposed worms. Electron-dense ferritin-containing iron particles have been reported from mammalian lysosomes that phagocytose red blood cells, and the transformation or incorporation of damaged mitochondria into cytolysosomes is a common method by which mitochondria are eliminated from the body (Muirden and Rogers, 1978; Ghadially 1982). In *U. caupo*, these putative sulfide oxidizing bodies may scavenge mitochondria or coelomic fluid heme and secondarily use it for sulfide detoxification by binding sulfide or accelerating sulfide oxidization in a manner similar to that reported for the worms' coelomic fluid heme compound, hematin (Powell and Arp, 1989; Arp, 1991; Arp *et al.*, 1992; Menon and Arp, 1992b). Theoretically, the binding or oxidation of sulfide to nontoxic forms of sulfur in peripheral body tissues would prevent the penetration of this aerobic poison to vital muscle tissues. The relationship between heme iron, mitochondria, and lysosomal sulfide oxidizing bodies in *U. caupo* is currently under investigation.

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